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REVIEW

Molecular Tools for the Detection and Deduction of Azole Antifungal Drug Resistance Phenotypes in *Aspergillus* Species

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SUMMARY The incidence of azole resistance in *Aspergillus* species has increased over the past years, most importantly for *Aspergillus fumigatus*. This is partially attributable to the global spread of only a few resistance alleles through the environment. Secondary resistance is a significant clinical concern, as invasive aspergillosis with drug-susceptible strains is already difficult to treat, and exclusion of azole-based antifungals from prophylaxis or first-line treatment of invasive aspergillosis in high-risk patients would dramatically limit drug choices, thus increasing mortality rates for immunocompromised patients. Management options for invasive aspergillosis caused by azole-resistant *A. fumigatus* strains were recently reevaluated by an international expert panel, which concluded that drug resistance testing of cultured isolates is highly indicated when antifungal therapy is intended. In geographical regions with a high environmental prevalence of azole-resistant strains, initial therapy should be guided by such analyses. More environmental and clinical screening studies are therefore needed to generate the local epidemiologic data if such measures are to be implemented on a sound basis. Here we propose a first workflow for evaluating isolates from screening studies, and we compile the MIC values correlating with individual amino acid substitutions in the products of *cyp51* genes for interpretation of DNA sequencing data, especially in the absence of cultured isolates.

KEYWORDS *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus terreus*, *Aspergillus niger*, azole drug resistance, diagnostics, *cyp51A*, *cyp51B*, *cyp51C*, efflux, tandem repeats

INTRODUCTION

Inhalation of *Aspergillus* spores, primarily of the species *Aspergillus fumigatus*, might result in different clinical manifestations. Whereas sequelae are usually absent in a healthy host, frequent exposure to spores might foster the development of allergic bronchopulmonary aspergillosis in individuals with an atopic disposition. Cavitary lung disease, e.g., following tuberculosis, might predispose individuals to aspergilloma. Chronic necrotizing aspergillosis is predominantly observed in patients who suffer from chronic lung disease or mild immunosuppression. Invasive pulmonary aspergillosis is the most dangerous and life-threatening clinical manifestation and might result from *Aspergillus* exposure in heavily immunosuppressed patients (1). In fact, *Aspergillus* spp., primarily *A. fumigatus*, are the most common emerging fungal pathogens, especially in patients with malignant hematological diseases who are undergoing intensive chemotherapy or after allogeneic hematologic stem stell transplantation. Consequently, prolonged neutropenia ($<$500 cells/μl for more than 10 days) has been identified as a major risk factor (1).

Over time, several azole-based antimycotic drugs have been developed, among which itraconazole (ITZ), voriconazole (VRZ), posaconazole (PSZ), and (lately) isavuconazole (ISAZ) are mainly used for the treatment and/or prophylaxis of aspergilloses (Fig. 1). Azoles are steric inhibitors of sterol 14α-demethylase enzymes catalyzing a critical step in ergosterol biosynthesis (Fig. 2A and B).

The infection-related mortality rates are still exceptionally high, despite recent improvements in prophylaxis, early diagnosis, and antifungal treatment of the disease (2). The incidence of azole resistance in *Aspergillus* spp. has increased over the past years, additionally jeopardizing the outcomes for high-risk populations by failure of azole-based prophylaxis and first-line treatment of invasive aspergillosis.

Starting with the initial observation in a Dutch strain collection that over the 1990s numbers of azole-resistant *A. fumigatus* (ARAf) had increased unproportionally due to a single resistance allele (3), the same has now been demonstrated on a global level (4). Different patterns of resistance are seen, with multiazole and pan-azole resistance being more common than resistance to a single triazole. A variety of mutations in the coding region of *cyp51A* (Fig. 2C), a gene encoding a sterol 14α-demethylase in *A. fumigatus*, as well as tandem repeats of 34, 46, and 53 bp upstream in its promoter region (Fig. 2E), have been found to confer, or at least to correlate with, various degrees of drug resistance. Today, the most frequently observed resistance allele consists of a 34-bp tandem repeat in the promoter region of *cyp51A* combined with the L98H substitution (TR_{34}/L98H). This allele confers high ITZ resistance and various levels of cross-resistance to other azoles. Together with strains carrying a TR_{46} allele in conjunction with Y121F and T289A exchanges, such strains are thought to spread globally through the environment (5), potentially propagated by the use of 14α-demethylase inhibitor (DMI) fungicides in agriculture. In fact, for many plant-pathogenic fungi, similar resistance mechanisms toward agricultural azole-based fungicides are observed (6–10), and their spread to the level of complete replacement of susceptible strains in some regions (11, 12) is a much discussed topic in phytopathology. The best-studied example is the wheat pathogen *Zymoseptoria tritici*, in which the *cyp51B* paralog has evolved several resistance mutations in both promoter and coding regions as a result of azole selection (for an overview, see reference 12).

Resistant *Aspergillus* strains present in the environment are thought to exogenously colonize or infect susceptible hosts. These observations are of significant concern, as exclusion of azole-based antifungals from prophylaxis or first-line treatment of invasive aspergillosis dramatically limits treatment options (4). A careful reevaluation of remaining management options for invasive aspergillosis caused by azole-resistant strains by

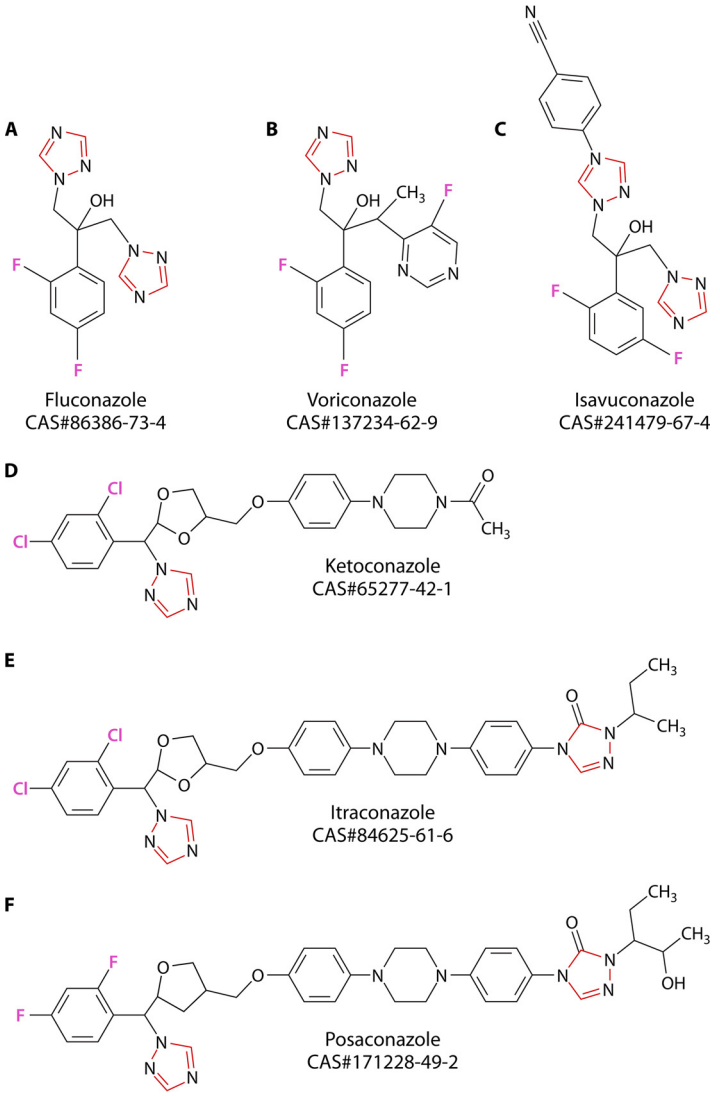

FIG 1 Chemical structures of clinically used azole antifungals. Azoles are characterized by five-atom heterocycles which contain at least one nitrogen atom (red). Compounds containing moieties with two nitrogen atoms are called diazoles, and those with three nitrogen atoms are called triazoles. Recently marketed antifungals contain one (B and D) or more (A, C, E, and F) triazole moieties and a benzene ring substituted with fluorine (A to C and F) rather than chlorine (D and E). Triazole antifungals are derivatives of either fluconazole (A to C) or ketoconazole (D to F) as the lead compound. This correlates with cross-resistance phenotypes observed in clinical and environmental isolates (see the text).

an international expert panel suggested that, in initial therapy, the environmental prevalence of azole-resistant strains should be considered in areas where the prevalence reaches 10% (13, 14). For example, this may be done by combining VRZ with an echinocandin or liposomal amphotericin B (13, 15).

In about 20 to 50% of azole-resistant clinical *A. fumigatus* isolates, no mutations within the *cyp51A* locus are observed (16–18); therefore, additional pathways and factors that confer secondary resistance must exist. In this context, only the HAP complex member *hapE* (CCAAT binding complex [CBC]) has been implicated in clinical ARAf so far (19, 20). Additionally, drug efflux has been discussed to contribute to resistance (17, 21, 22). Hence, different azole resistance patterns may be multifactorial, and this must also be considered when encountering isolates with an altered *cyp51* sequence (23).

The molecular detection of genetic alterations leading to azole resistance is gaining importance, given the poor culture-based diagnostic yield for clinical specimens from

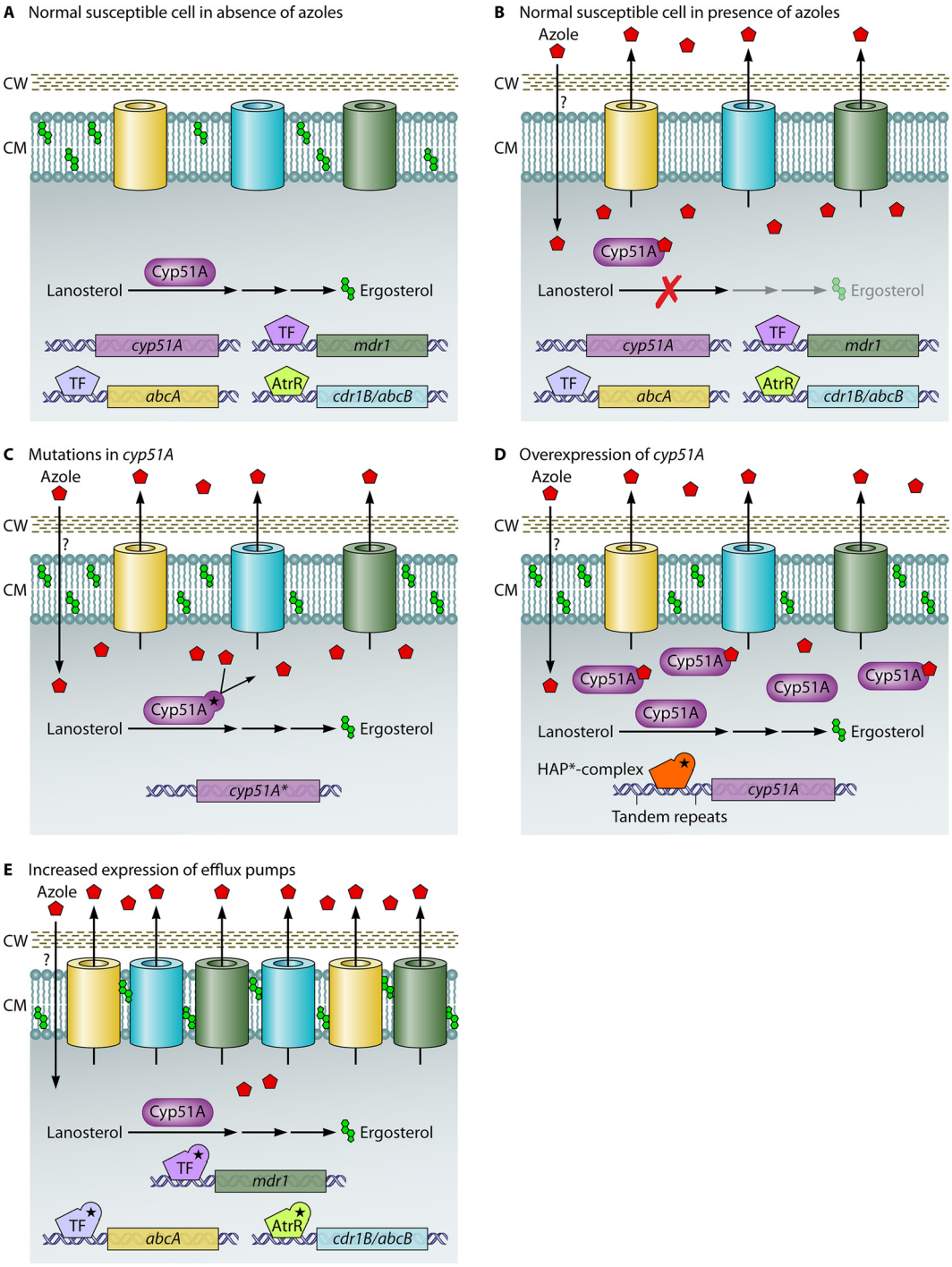

FIG 2 Azole antifungal drug resistance mechanisms in fungal cells. (A) *A. fumigatus*, including the cell wall (CW) and the cytoplasmic membrane (CM), in the absence of azoles. Cyp51 activity is required for the biosynthesis of the membrane compound ergosterol (green). The *abcA*, *cdr1B*, and *mdr1* genes encode efflux pumps, which are localized in the plasma membrane. These genes are regulated by different transcription factors (TF), such as AtrR. (B) In the presence of azoles, some drug molecules can be pumped out through efflux pumps, but intracellular levels are sufficient to inhibit Cyp51A, resulting in decreased amounts of ergosterol in the membrane. (C) Mutations in *cyp51A* (asterisk) can reduce target binding of the antifungal drug, therefore conferring resistance. (D) Overexpression of the drug target Cyp51A can be mediated by mutations in the HAP complex (HAP*) or by different kinds of tandem repeats within the promoter region of *cyp51A*. (E) Increased expression of efflux pumps, such as AbcA, Cdr1B, and Mdr1, increases the drug tolerance of *A. fumigatus*. Possible mechanisms for increased transcription may be gain-of-function mutations in the regulating transcription factors (TF*, AtrR*).

hematological patients. From a clinical point of view, azole resistance is associated with therapeutically relevant azole treatment failure that is increasingly reported and plays a more and more important role, especially in hematology and intensive care unit (ICU) patients, leading to high mortality rates (24–27).

There is an ongoing debate as to how epidemiologic thresholds should be generated and to what degree laboratory findings of azole resistance in *A. fumigatus* outside invasive aspergillosis should be reported to clinicians (28). Nevertheless, it is evident that medium- to large-scale environmental and clinical screening studies are urgently needed to generate epidemiologic data to reassess clinical treatment options on local or national levels. Molecular analysis of such isolates is not readily available to most diagnostic laboratories and is far from being standardized. Therefore, we outline here a stepwise workflow for evaluating isolates from such screening studies to build up local epidemiologic data (Fig. 3). Furthermore, we created an annotated reference sequence for easy identification of known polymorphisms in *A. fumigatus* Cyp51A, in accordance with their recently proposed unified nomenclature (29), and compiled the MIC values correlating with individual Cyp51A amino acid substitutions. This should aid in the interpretation of DNA sequencing data, especially in the absence of a culture isolate.

SCREENING FOR AZOLE-RESISTANT *ASPERGILLUS* STRAINS

Clinical guidelines, e.g., by the Infectious Diseases Society of America (IDSA) (30, 31), do not specify laboratory testing procedures but rather reference the general literature on the isolation of *Aspergillus* from respiratory tract specimens (32, 33).

Only recently has it been recommended "...to test different, up to five, colonies as different azole susceptibility phenotypes might be present in a single culture" (13). Indeed, rapid conidial dispersion is a key feature of *Aspergillus* spp., especially *A. fumigatus* (34). This allows the fungi to rapidly colonize fresh materials and, presuming a certain selective pressure in the environment, also explains the rapid clonal expansion of resistance phenotypes (35) and makes mixed samples very likely. Dissemination of resistant isolates is also directly propagated by human activities (36).

A very educative experiment highlighting how easily conidia of *A. fumigatus* are moved through the air was conducted by Kwon-Chung and Sugui (34). In their setup, fresh agar plates were kept next to a sporulating *A. fumigatus* culture plate. Compared to growth in the same setup with *A. nidulans*, growth was observed on the fresh plates in a very rapid fashion, despite all attempts to keep the environment free of air movement. The lecture to be taken here with respect to laboratory work is that, when handling aspergilli, especially *A. fumigatus*, working in the best possible sterile environment free of air movement is highly advisable to avoid cross-contamination between samples and/or cultures. In addition, both environmental and clinical samples may contain susceptible along with resistant fungal material (37, 38). This leads to a reduced detection rate for resistant strains under regular culture conditions in cases where susceptible fungal cells pose the majority of the inoculum. In such cases, additional use of screening agars with included antifungal drugs may be highly beneficial to suppress the growth of most susceptible strains (Fig. 3). For some samples, this may lead to the detection of a single resistant colony only. This emphasizes the need to reduce the number of cross-contaminating conidia from previously cultured samples. Best practice will physically separate the primary culture from any subsequent handling of cultures, especially those with resistance phenotypes.

Standardized protocols for screening of environmental samples do not yet exist. This may be due partially to the heterogeneity of such "environmental samples," which may encompass dry soil (e.g., see references 39 to 43), air samples (35, 44–46), plant material (43), or simply surface swabs (46). For soil, rigorous vortexing in NaCl (43) and plating of the supernatant following settling of the soil debris have been used. Addition of 0.5% (wt/vol) saponin facilitated the extraction of highly hydrophobic material, i.e., *A. fumigatus* conidia (39, 40). To restrict the growth of most potential contaminants, incubation at elevated temperatures (>43°C) can be used, as *A. fumigatus* is highly

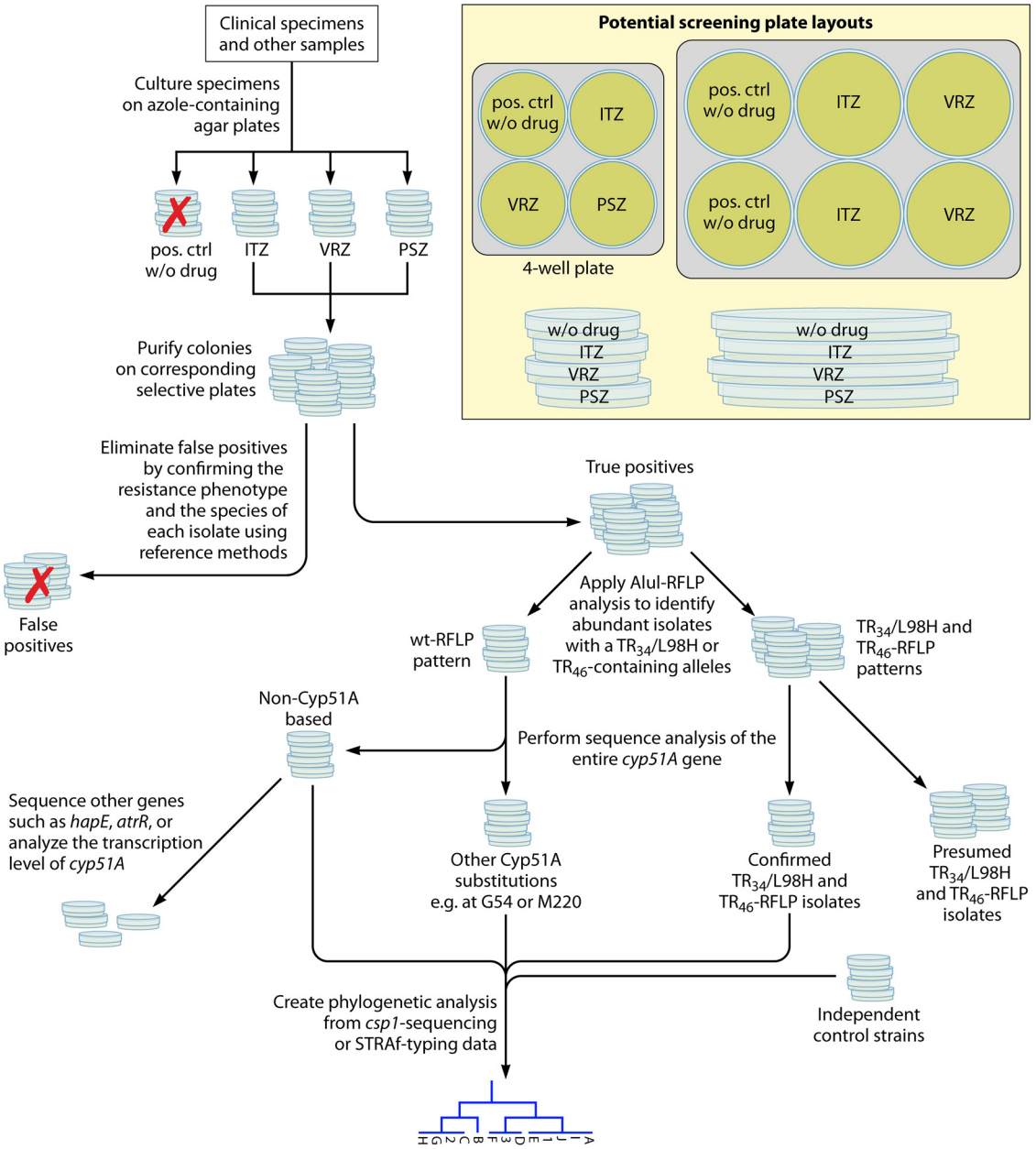

FIG 3 Potential sampling workflow for ARAf screening studies. No standardized scheme for conducting screening studies is established yet, but combining several approaches proposed in the literature gives rise to an efficient workflow that eliminates false-positive results and yields robust numbers on the prevalence and phylogenetic cohesion of resistant isolates.

thermotolerant (47). However, especially environmental samples from tropical areas may still yield strong overgrowth with other thermotolerant species (our own unpublished observation).

Drug concentrations for screening agar plates used in the literature range from 0.5 μg/ml for PSZ (48, 49) to 1 to 4 μg/ml for ITZ or VRZ (39, 43, 48–50). At the lower end of the concentration range, a higher false-positive rate may be observed (39). When working with the goal of isolating *A. fumigatus*, specifically screening for fluconazole resistance is not necessary, as *A. fumigatus* is intrinsically resistant to this compound (51–53).

Different mutations leading to singly elevated resistances to particular drugs only are known (Tables 1 and 2). MIC values measured for isolates with altered azole susceptibility are cumulated in Tables 1 and 2. MIC values for tests done according to

TABLE 1 Levels of drug resistance conferred by mutations in *A. fumigatus cyp51A*, using EUCAST breakpoints[a]

	No. of isolates with MIC (μg/ml)																																		
	Itraconazole											Voriconazole												Posaconazole											
	S		I	R								S					I	R						S		I	R								
Mutation	<1	1	2	4	>4	8	>8	16	>16	32	>32	<0.125	0.125	0.25	0.5	1	2	>2	4	>4	8	>8	16	>16	<0.125	0.125	0.25	0.5	1	2	4	>4	>8	>16	>32
TR_{34}/L98H			7	4	18	13	32	13	55	7	7			1	3	23	42		62		18		5	2		10	13	54	53	20				1		
TR_{34}/L98R									1								1											1								
TR_{34}/L98Y									1							1	1											1								
TR_{34}/L98Q									1								1											1								
TR_{34}/L98I									1							1												1								
TR_{34}/L98H/S297T/F495I									6							2	3		1									3	3							
TR_{46}/Y121F/T289A	5	2	2			1	3	1	3							1				3	1	5	3	4		2	3	4	3	2				1		
TR_{46}/Y121F/T289A/G448S									1															1										1		
TR_{46}×3/Y121F/M172I/ T289A/G448S									1															1					1							
F46Y/M172V/N248T/D255E									1								1												1							
G54E									8		1	1		2	3	1	1	1										1	7	1						
G54R						1		2			1			2				2										2	1	1						
G54R/M220T							1								1															1						
G54W									2		1				2	1																1		1		1
Y121F	1																1									1										
P216L							1		4						1	2	2											1	4							
F219C											1				1											1										
F219I									6							1			2		3							1						5		
M220I					8		1				1				1	1	4	3		1							1	1	1		7					
M220K					3											1	1		1												2					
M220L							3								2	1													1				2			
M220T							3			1					3				1						1		2	1								
M220V											1								1											1						
M220R									1												1														1		
Y431C						2													2										2							
G432A				1													1											1								
G434C							1												1										1							
G448S							2												1			1							1							

[a]Values are cumulative numbers of isolates with the respective MIC values reported in the literature, stratified according to clinical EUCAST breakpoints (133). S, susceptible; I, intermediate; R, resistant.

TABLE 2 Levels of drug resistance conferred by mutations in *A. fumigatus cyp51A*, using CLSI breakpoints[a]

	No. of isolates with MIC (μg/ml)																																		
	Itraconazole											Voriconazole														Posaconazole										
	S		I	R								S					I	R								S					I	R				
Mutation	<1	1	2	4	>4	8	>8	16	>16	32	>32	<0.125	0.125	0.25	0.5	1	2	>2	4	>4	8	>8	16	>16	<0.125	0.125	0.25	0.5	1	2	4	>4	>8	>16	>32
TR_{34}/L98H		11				2	14	12	59							5	28	5	24		23	6	7	5		1	3	50	21	12	2		4			
TR_{46}/Y121F/T289A	8	2	2	8				1	6													9		18			4	11	7	5						
G54E			2	2		1	8	7	5				1	15	7	1											2	5	5	4						
G54R			2				2	4	7				4	3	5		1		1							1			5	3				1		
G54V							4	1						4		1												2		1						
G54W		2	2				2		7				3	5	2	2	1							1		1	1			1			6	4		
G54E/I266N			4	2		1									5	1	1									2	5			1						
G138C							2		2												3		1						1		2			1		
G138S									1														1							1						
F219I									1					1													1									
F219S									1								1													1						
M220I							1		8					1	4	3	1										1	6	2							
M220K							9	2	2							8	3												3	6	1						
M220R									3						1		2											1		2						
M220T							3	1							1	2											1									
M220V							8	5	1						1	12	1											5	3							
I266N				1			1								2													2								
G448S	2			1				1	2						1	2					1		1	1			2		2		2					

[a]Values are cumulative numbers of isolates with the respective MIC values. Clinical breakpoints for results obtained with the CLSI methodology are not available; the values for S/I/R classification used here were adopted from reference 190.

the European Committee on Antimicrobial Susceptibility Testing (EUCAST) protocols were taken from previous references (16, 18, 24, 26, 38, 42, 44, 49, 54–81), and MIC values for tests done according to the Clinical and Laboratory Standards Institute (CLSI) protocols were taken from other previous references (3, 35, 43, 45, 50, 82–107).

Clinically used antifungal triazole compounds fall into two distinct structural classes (Fig. 1), which largely correlate with cross-resistance phenotypes: in *A. fumigatus* isolates with mutations in the *cyp51A* gene, cross-resistances within the substance pairs ITZ/PSZ and VRZ/ISAZ are most strongly correlated (16, 108). ISAZ and its azonium sulfate derivative for oral formulations have become an alternative for the therapy of invasive aspergillosis in patients with VRZ intolerance and seem to be more favorable than VRZ regarding drug-drug interactions (109).

In light of this, the use of at least VRZ along with ITZ as screening drugs is recommended to cover the range of currently known resistance phenotypes. Where available, extension of screening to the clinically relevant substances PSZ and even ISAZ may become advisable in the future.

SPECIES IDENTIFICATION OF *ASPERGILLUS*

Exact species determination of molds by morphology, including that of *Aspergillus* spp., is a nontrivial issue (110). Molds can be slow to generate species-specific morphological traits allowing their discrimination, and they often require rather specific conditions to do so (111). On Sabouraud's agar plates, *Aspergillus* colonies appear as dense fields of conidiophores of various colors. Of the species discussed here, *A. flavus* appears yellowish to greenish, *A. fumigatus* and its close sibling species dark blue-green, *A. nidulans* green to dark yellow, *A. niger* black, and *A. terreus* light brownish (112). Due to the absence or delay of conidium formation, *A. fumigatus* may vary in its visual appearance to nearly white ("albino phenotype") (16, 111, 113). In addition, exact species boundaries are still not defined in all cases, and classification will ultimately require sequencing of several loci (e.g., internal transcribed spacer [ITS], *tub1*, *benA*, and *cmd* [114, 115]). Matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometric species identification was recently extended to clinically important molds (116–118). In our own experience, commonly isolated species can readily be differentiated by using the commercial databases, and in cases where isolates with a common look (e.g., the bluish appearance of *A. fumigatus*) do not give rise to identification via MALDI-TOF, they turn out to be cryptic species not yet included in the scheme (119).

SUSCEPTIBILITY TESTING PROCEDURES FOR *ASPERGILLUS* SPP.

There are several assays for susceptibility testing of *Aspergillus*, such as broth macrodilution (120), broth microdilution (121–123), and disk diffusion (124, 125) assays, Etest/MIC strip kits (126–128), and other commercial kits (129). Each method has distinct advantages and disadvantages; for example, macro- and microdilution assays are laborious and time-consuming, whereas Etest is easy to perform but expensive when used on a larger scale, and agar diffusion is inexpensive but not able to indicate exact MIC values (120).

Subsequent retesting and quantitative determination of MIC values by use of a reference method are also indicated to determine possible cross-resistance between compounds. In larger studies, two broth microdilution reference methods are mainly used for susceptibility testing: the CLSI M38-A2 protocol (121) and the EUCAST E.DEF 9.1 protocol (123, 130). These two standard methods are highly reproducible, and the MICs can be used for therapeutic selection of antifungal drugs as well as monitoring of antifungal drug susceptibility during treatment (120).

These two methods differ in several critical technical points, such as the amount of fungal inoculum (4×10^4 to 5×10^4 CFU/ml as determined by use of a spectrophotometer for the CLSI method and 2×10^5 to 5×10^5 CFU/ml as determined by use of a hemocytometer for the EUCAST method). In addition, the EUCAST method uses a higher percentage of glucose (2%) in the test medium, which facilitates an increased

growth rate and specifies the use of flat-bottomed microtiter plates (120). It should be noted that both protocols have limitations in testing amphotericin B, which shows only a very narrow range of MIC values (0.5 to 2 μg/ml), as well as PSZ and the echinocandins (131). Because of their technical differences, the methods generate different absolute MIC values (131, 132), and consequently, divergent clinical breakpoints have been established by both EUCAST and CLSI (121, 133). EUCAST provides clinical breakpoints for antifungal agents for the interpretation of susceptibility testing results for fungi. Among molds, species-related clinical breakpoints have been determined only for *A. fumigatus*, *A. flavus*, *A. nidulans*, *A. niger*, and *A. terreus* (133). Using the EUCAST breakpoint table on these species, resistant isolates against the defined antifungal agents can be identified and subsequently investigated for mutations. There are no clinical breakpoints available from the CLSI, and therefore epidemiological cutoff values must be used (121).

In clinical studies, the results of *in vitro* susceptibility testing of *A. fumigatus* isolates did not necessarily correlate with the expected outcome of antifungal therapy in infected patients (123). From these studies, the "90-60 rule" was established, which implies that patients with infections caused by an *in vitro* susceptible strain will respond to the antifungal treatment in approximately 90% of cases, whereas patients suffering from infections caused by resistant isolates will respond in only about 60% of cases (120, 134). Therefore, although broth microdilution susceptibility screening is valuable for the selection of the best therapeutic agent, reliable prediction of the clinical outcome in a patient during antifungal therapy is still cumbersome (120, 130, 131). For these reasons, the combined use of molecular approaches to identify resistance and the phenotypic susceptibility testing described above may improve and guide successful treatment.

EVOLUTION OF *cyp51* GENE FAMILIES

The major targets of azole antifungal drugs are cytochrome P450-type enzymes with sterol 14α-demethylase activity. Inhibition of these enzymes leads to the formation of aberrant sterols, which do not complement the function of ergosterol in the plasma membrane and have cytotoxic and eventually fungistatic properties (135). The corresponding genes are called *cyp51* in molds and *ERG11* in ascomycetous yeast. P450 cytochromes are an evolutionarily ancient protein family and have undergone significant expansion and functional divergence across the entire phylogenetic tree. In molds, this is signified by at least three different lineages of paralogous genes, namely, *cyp51A*, *cyp51B*, and *cyp51C*.

Transcript and therefore protein abundance can be a direct function of gene copy number ("gene amplification" or "gene dosage effect"). If another copy of a *cyp51* gene is introduced, this can have a direct effect on drug resistance. For example, heterologous expression of a second *pdmA* copy in *A. nidulans* leads to strains that are selectable on azole-containing agar (136). This demonstrates that gene amplification may also contribute to tolerance, possibly as a prerequisite step to the expansion of *cyp51* gene families.

However, in evolutionary time spans, such duplicated genes will be fixed in a population only when they diverge in function (137). Paralogous genes may be lost and even may reemerge in populations (138).

Among *Aspergillus* spp., section Fumigati species contain *cyp51A* and *cyp51B* (139). Heterologous expression as well as biochemical testing has revealed differences between *A. fumigatus* Cyp51A and Cyp51B; despite identical substrate preferences and identical sensitivities against ITZ, VRZ, and PSZ, the isoenzymes showed differential tolerance of fluconazole, which was significantly increased for Cyp51A compared to that of Cyp51B (53, 140, 141). On comparing the Cyp51 protein sequences of molds to those of yeasts, e.g., Erg11 of *Candida albicans* (51), for which a multitude of substitutions have been brought forward in the context of azole resistance (e.g., see references 142 and 143), it becomes evident that their phylogenetic relationship is far from clear and that direct knowledge transfer to resistance in molds is difficult, at best. The proper

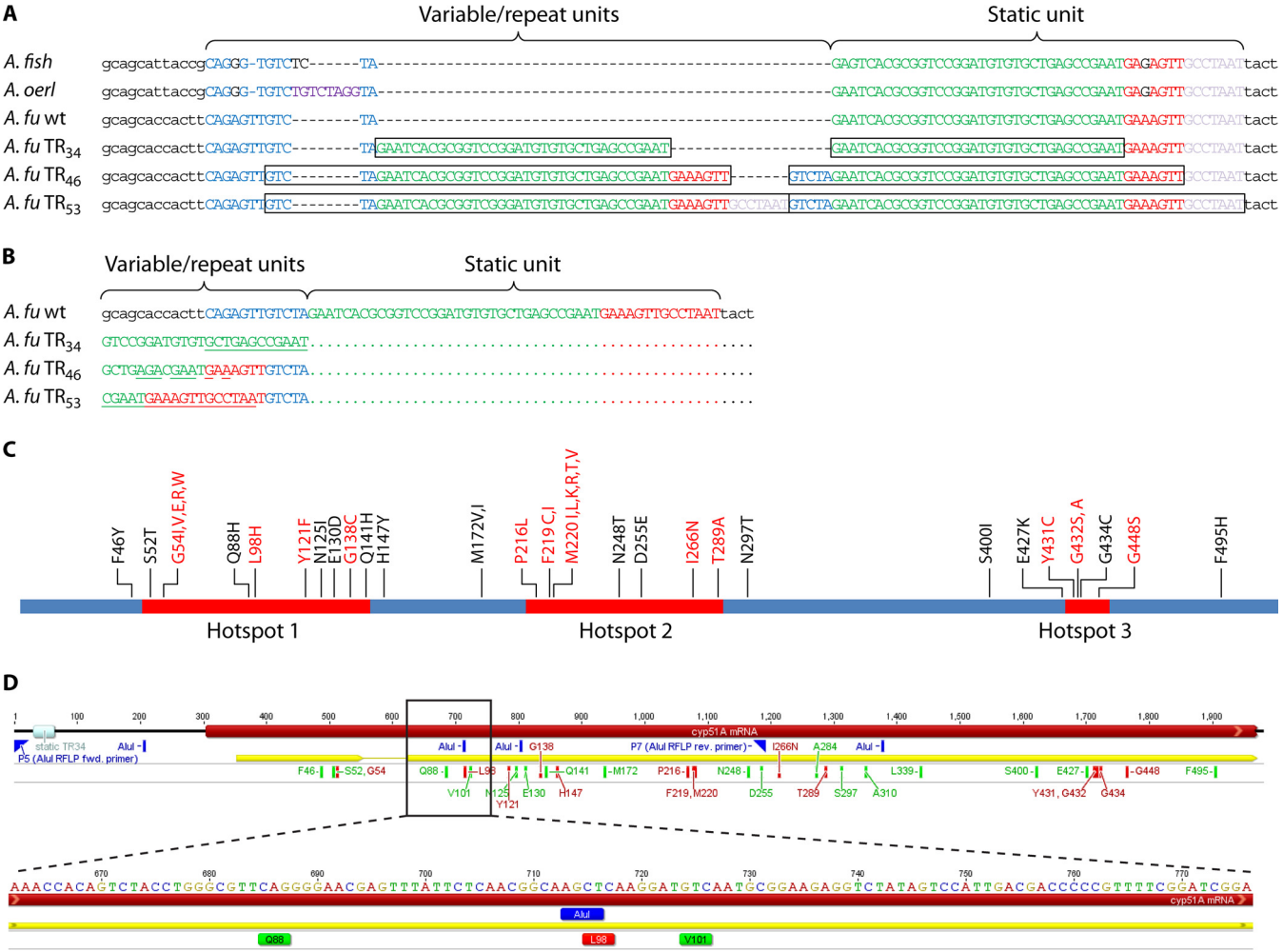

FIG 4 Mutations in *A. fumigatus cyp51A* and its promoter region. (A) Alignment of the TR_{34} (taken from isolate 168 [16]), TR_{46} (taken from an environmental isolate [39]), and TR_{53} (sequence taken from a Columbian isolate [41; P. LePape, personal communication]) promoter alleles with the wild-type sequences from *A. fumigatus* (*A. fu*), *A. fischeri* (*A. fish*), and *A. oerlinghausenensis* (*A. oerl*) (119). Green, TR_{34} repeat unit; blue or red, 5′ or 3′ region and respective repeat sequences probably stemming from there. Black uppercase residues indicate sequence divergence in the repeat region, lowercase residues indicate adjacent residues, and black boxes indicate TR_{34}, TR_{46}, and TR_{53} repeats. (B) In sequencing data, these three different types of *A. fumigatus* can easily be differentiated by the 5′ upstream sequence preceding the static repeat unit: sequences different from the wild-type sequenc are indicated by underscored nucleotides. (C) Known amino acid substitutions in Cyp51A. Red, known resistance-conferring substitutions; black, substitutions present in the population, probably without an effect on drug susceptibility. (D) *cyp51A* gene features visualized by use of sequence analysis software (Geneious R10). The data are available as a .gb annotated file or as a Word document in a community-editable form from https://github.com/oliverbader/Aspergillus_fumigatus_cyp51A.

paralog to *C. albicans ERG11* is probably *cyp51B* (29). To complicate matters, due to differences in expression patterns (Cyp51B is expressed constitutively, and Cyp51A is inducible), Cyp51A is believed to be the major player in azole resistance in *A. fumigatus*.

Among section Flavi species, at least for *A. flavus* and *A. calidoustus*, the additional paralog *cyp51C* is present, and in *A. calidoustus cyp51A* has been lost (144). In these species, Cyp51C appears to be the key player (see below).

RESISTANCE THROUGH TRANSCRIPTIONAL MODULATION OF *cyp51* GENES

To date, three different azole resistance-associated variations of the *cyp51A* promoter (Fig. 4A) are known for *A. fumigatus*. Most frequently, two tandem repeats, of 34 and 46 bp, have been described to occur 279 bp upstream of the coding region (84, 98, 145). The TR_{34} promoter has been found only with the L98H exchange conferring high ITZ resistance, and the TR_{46} promoter has been found in conjunction with the Y121F and T289A exchanges in intermediate to highly VRZ-resistant isolates. Additionally, a 53-bp repeat has been found in isolates that are cross resistant to VRZ and ITZ, without

any further Cyp51A alterations (41, 145). Most recently, isolates with up to three or four copies of the TR_{46} repeat were identified (81). Changes in the promoter region must be interpreted in the context of gene expression levels (Fig. 2D). For the TR_{34} promoter, a 2-fold higher activity in a reporter assay (146) and an 8-fold higher *cyp51A* gene expression level (84) have been reported. However, this remains untested so far for any other promoter variants. Molecular studies of the altered *A. fumigatus cyp51A* promoter have identified opposing actions of the CCAAT binding complex (CBC), which is involved in the regulation of many genes in eukaryotes by binding CCAAT sites (19, 147, 148), and the HAP complex, an element of the sterol response pathway (20). Genome sequencing of matched resistant and susceptible isolates also identified a mutation in *hapE*, introducing the P88L amino acid exchange, which was shown to be responsible for the azole resistance phenotype (19). Detailed molecular studies of the HAP complex have found it to interact with the *cyp51A* promoter at the repeat elements found in drug-resistant isolates, there opposing the action of the sterol response factor SrbA (20). The P88L substitution leads to a significant loss of binding activity, in turn leading to unopposed action of the sterol biosynthesis response pathway, upregulating *cyp51A*, among other sterol biosynthesis genes. As it has been reported only once, the number of clinical isolates containing the P88L substitution in HapE appears not to be very high, or at least underinvestigated.

Outside *A. fumigatus*, data on *cyp51* promoter alterations are scarce. Only in *Aspergillus* (*Neosartoriya*) *fischeri* and *Aspergillus oerlinghausense*, the two known species most closely related to *A. fumigatus*, can a similar to identical 34-bp core repeat region readily be identified in the respective promoters of the corresponding *cyp51A* genes (Fig. 4A). In *A. oerlinghausense*, a short insertion upstream of the core repeat correlates with the decreased azole susceptibility in the only two existing isolates of this new species (119), albeit molecular confirmation for this observation is currently lacking. The 1-kb regions flanking the three *cyp51* paralogs of resistant and susceptible *A. flavus* isolates investigated (149) did not deviate from each other, but further upstream a short 4-bp deletion was found in two resistant strains (149). The role of this deletion remains unclear. In sequencing data for *A. fumigatus cyp51A*, the type of repeat can easily be identified by the first 10 bp preceding the static 34-bp repeat unit (Fig. 4B).

RESISTANCE-CONFERRING ALTERATIONS WITHIN Cyp51 PROTEINS

Mair et al. (29) recently suggested the use of a positional nomenclature for fungal resistance mutations to allow easier transfer of knowledge on orthologous proteins of different species. Based on a preformed alignment of the amino acid sequences, the respective number in a reference is given instead of the residue number of the affected amino acid itself. For Cyp51A-orthologous proteins, the reference was suggested to be Cyp51A of *A. fumigatus*, and for Cyp51B-orthologous proteins, the reference is Cyp51B of *Zymoseptoria tritici* (anamorph of *Mycosphaerella graminicola*). For Cyp51C, no suggestion has yet been made; for the purpose of this review, we therefore name Cyp51C of *A. flavus* as the reference. The positional nomenclature is used below and summarized in Table 3.

Aspergillus fumigatus

In approximately 50 to 80% of the clinically observed ARAf isolates, resistance is attributable to amino acid exchanges within the Cyp51A protein (16–18, 95). Cyp51B substitutions seen in the population apparently do not contribute to resistance. Similarly, only a subset of Cyp51A substitutions present in the population lead to resistance against azoles (Fig. 4C). Notably, several synonymous (silent) mutations in *cyp51A* are also known, e.g., at the L358 or C454 codon (82) or the L77, R65, or E356 codon (150). These are interesting in a phylogenetic context but have no relevance for deduction of resistance phenotypes. On encountering nonsynonymous mutations in sequencing data, these must be discriminated from allelic variants without any effects on azole susceptibility. Several substitutions (e.g., F46Y, H147Y, M172V, N248T, N248K, D255E, D343N, E427K, and G434C) seem not to be involved in azole resistance, as they are also

TABLE 3 Cyp51 substitutions in non-*A. fumigatus* organisms and their correspondence to the reference positions used in the text

Organism	Reference position[a]			Observed substitution		
	Gene	Position[b]	Reference	Gene	Substitution	Reference
A. flavus	*Afcyp51A*	Y68	29	*cyp51A*	Y132N	160
	Afcyp51A	K133	29	*cyp51A*	K197N	160
	Afcyp51A	NA		*cyp51A*	A205T	149
	Afcyp51A	D280	29	*cyp51A*	D282E	160
	Afcyp51A	M286	29	*cyp51A*	M288L	160
	Afcyp51A	T470	29	*cyp51A*	T469S	160
	Ztcyp51B	H430	29	*cyp51B*	H399P	160
	Ztcyp51B	A453	29	*cyp51B*	D411N	160
	Ztcyp51B	T496	29	*cyp51B*	T454P	160
	Ztcyp51B	NA		*cyp51B*	T486P	160
	Afcyp51C	M54	Proposed here	*cyp51C*	M54T	149
	Afcyp51C	S196	Proposed here	*cyp51C*	S196F	161
	Afcyp51C	S240	Proposed here	*cyp51C*	S240A	149, 162
	Afcyp51C	D254	Proposed here	*cyp51C*	D254N	149
	Afcyp51C	I285	Proposed here	*cyp51C*	I285V	149
	Afcyp51C	Y319	Proposed here	*cyp51C*	Y319H	149, 191
	Afcyp51C	A324	Proposed here	*cyp51C*	A324P	161
	Afcyp51C	N423	Proposed here	*cyp51C*	N423D	161
	Afcyp51C	V465	Proposed here	*cyp51C*	V465 M	161
A. terreus	*Afcyp51A*	M220	29	*cyp51A*	M217I	165
A. niger	*Afcyp51A*	K230	29	*cyp51A*	R228Q	166
A. tubingensis	*Afcyp51A*	L21	29	*cyp51A*	L21F	166

[a]Accession numbers for reference sequences are as follows: *A. fumigatus cyp51A*, AF338659; *Zymoseptoria tritici* (anamorph of *Mycosphaerella graminicola*) *cyp51B*, AY253234; and *cyp51C*, AKQ20794.1.

[b]NA, not applicable (the respective residue was not found at this position in publicly deposited *A. flavus* sequences).

found in different combinations in azole-susceptible isolates (56, 62, 70, 75, 82, 87, 151), and these sites are mostly localized at the protein surface (152). Among these, isolates harboring the multiply substituted *cyp51A* F46Y/G89G/M172V/L358L/E427K/C454C allele (18, 56) appear to form a separate phylogenetic subgroup rather than grouping with strains with acquired resistance. Nevertheless, these substitutions do occur alongside those with relevance for reduced antifungal drug susceptibility, e.g., the TR_{34}/L98H substitution (Fig. 4 and Table 2), likely where the original resistant strain crossed with another isolate of the population (153, 154).

The Cyp51A-I266N substitution reported from Japan (90) can probably be discarded as an interpretation error, as the reference sequence already has an asparagine (N) residue at this codon and it was not observed in subsequent studies (155).

Azole-based inhibitors of Cyp51 interact with the protein by complexing the heme group located in the catalytic center (152). For amino acid substitutions located along the periphery of the protein (e.g., S52T, Q88H, N125I, Q141H, S297T, or F495H), homology modeling suggests that they do not relevantly change its functionality (82, 152). Consequently, residues where substitutions lead to azole resistance, e.g., G54 (57, 83, 90, 103), G138 (56, 75, 104), or M220 (56, 82, 99), are mainly located close to the opening of one of the two ligand access channels of the protein, blocking the docking of most azole molecules (82, 152). Similarly, the L98 residue is located on a highly conserved, loop-forming, arch-like structure affecting the entry to the ligand access channel (82).

For several of the polymorphisms seen in antifungal drug-resistant isolates, the resistance-conferring nature of the particular substitution has been confirmed on a molecular level: biochemical testing of purified recombinant *A. fumigatus* Cyp51A-M220K and Cyp51A-G54W expressed in *Escherichia coli* (141) showed inhibition kinetics that would be expected from the respective clinical isolates' phenotypes, namely, a strong reduction in inhibition by ITZ and PSZ but not by VRZ. Interestingly, the G54W mutation does occur in both isolates with increased and those with susceptible MIC values (Tables 1 and 2). This raises the question of whether this substitution is actually relevant to clinical resistance, despite the fact that its contribution to reduced suscep-

tibility has been confirmed *in vitro* (141). This may also be an example of interactions with other factors, such as protein-protein interactions potentially dependent on the genetic strain background.

A very different situation was found for Cyp51A-L98H. In this case, inhibition of resistance was only marginally impaired, but the enzyme retained some residual activity at higher triazole concentrations, thus likely explaining its *in vivo* resistance phenotype (141). Structural modeling suggests that the L98H substitution is located at neither the substrate binding cleft nor the central heme group, but rather in a conserved surface arch, which would support these data (152).

The gold standard for *in vivo* testing of this is heterologous expression of a potential resistance-conferring allele in a susceptible host. In the case of *cyp51A* mutations from *A. fumigatus*, susceptible strains of the fungal model organism *Saccharomyces cerevisiae* and, more recently, *A. fumigatus* itself have been used.

S. cerevisiae is readily available for genetic studies and has also served as a host in several studies of azole resistance in several plant-pathogenic fungi (11). *S. cerevisiae* encodes only one Cyp51 ortholog, namely, Erg11. However, deletion of *ERG11* results in lethality, which is why complementation experiments were done in an *ERG11* tetracycline-repressible strain (140) or by a one-step gene replacement strategy (75). In these studies, introduction of the *cyp51A* or *cyp5B* gene was shown to complement the loss of the respective *S. cerevisiae* enzyme function.

Using a tetracycline-regulatable system, Alcazar-Fuoli et al. (156) were able to confirm the anticipated phenotypes for several mutations at positions G54 and M220: elevated ITZ MICs were found for G54E, G54V, G54W, and M220K mutants, and the M220K and M220I substitutions conferred VRZ resistance. The highest MIC values for PSZ were found for the G54W mutant, followed by the G54V, G54E, and M220K mutants. Using the gene replacement strategy, Albarrag et al. (75) confirmed decreased azole susceptibility for Cyp51A-G138C and Cyp51A-Y431C. Unexpectedly, the G434C mutant showed no resistance phenotype. The authors suggested that this method may not be suitable for alterations at the far C-terminal end of the protein, where interactions with as yet unknown binding partners may be the reason for reduced susceptibility in the original host.

Although a high degree of cross- and pan-azole resistance is seen, the quantitative degree of reduced drug sensitivity still varies between individual substitutions as well as compounds (Table 2). These effects may also be cumulative in cases where different promoter modifications and/or amino acid substitutions are combined in a single isolate. For *A. fumigatus*, these effects have been best investigated for the TR_{46}/Y121F/T289A allele. Isolates with this allele show high VRZ MICs along with reduced ITZ and PSZ susceptibilities (45). A single isolate with only the Y121F substitution showed only an intermediate VRZ MIC (157). Based on this, molecular dissection of this allele in an *A. fumigatus* expression system showed that the combination of TR_{46} with the Y121F mutation was sufficient to induce both high ITZ and VRZ resistances, while TR_{46} or the T289A mutation alone or in combination did not have a similar effect. Most strikingly, the strain arising from combination of the T289A mutation with the TR_{46}/Y212F allele was again less resistant to ITZ (158). Others have also found the TR_{46}/Y121F/T289A allele in combination with the G448S substitution in an isolate with pan-azole resistance (67). However, the G448S amino acid change can result in cross-resistance to VRZ and ITZ (159). The MIC values reported for the individual mutations are summarized in Tables 1 and 2, stratified by testing method.

Aspergillus flavus

Next to *A. fumigatus*, most other amino acid substitutions in Cyp51 proteins have been described for *Aspergillus flavus*. A key difference of this species from other *Aspergillus* species is the existence of a third paralog in *A. flavus*, namely, *cyp51C*. Substitutions have been observed in all three paralogs. Using the positional nomenclature (Table 3), these were at Y68, K133, D280, M286, and T470 in Cyp51A (149, 160),

	54						431	
	*						*	
A. fumigatus ***cyp51A***	TISY**G**IDPYK	QEVRSKLTA	ARMRSIYVDIINQRRLDGDKDSQ	EIAHMMITL	LEELYQEQLANLGP	TKEQENDE-VVD**Y**GYG	VNLGVILAT
	|| || |||	|||||||	||||||| |||| || ||	|||| ||||	||||||||||	| | |||||	|| || ||
A. flavus NRRL3357	TIHYG**M**DPYG	EEVR**S**KLTT	ARMR**S**IYIDIINKRRNAG**D**NVPE	EIAH**I**MITL	VEEL**Y**QEQL**A**NLER	PQEN**N**KD-DIVDYGYG	LNLA**V**IVAT
A. flavus AF70	.....**T**....	....**P**....	....**A**.....**N**....	....**I**....	....**Y**....**A**....	....**D**..-.....	....**V**....
	*	*	*	*	*	*	*
	54	196	240	254	285	319 323	423	465
A. oryzae RIB40	.....	.....	....A.....N....	.....	.....	.....-.....	.....
A. nomius	.....	.....	....A.....	.....	.....	.K..D..-.....	.....
A. lentulus	..S..I...K	Q.....A	.....V....Q..LD.....	....M....	L.....	AK.QEN.-EV.....	V.IG..L..
T. interdigitale	..S.....K	.....	....A..T...E...RE.GKDSQ	....M....	.....R.....GP	.NP.E.EE.M.....	V..T.....
T. tonsurans	..S.....K	.....	....A..T...E...RE.AKRNH	....M....	.....R.....PH	.NP.E.EE.M.....	V..T.....
T. rubrum	..S.....K	.....	S...A..TE..E...RE.AKRNH	....M....	L....R.....PH	.NTDEQEE.M.N....	V..T.....
T. soudanense	..S.....K	.....	S...A..TE..E...RE.AKRNH	....M....	L....R.....PH	.NTDEQEE.M.N....	V..M....T
M. canis	..R..L...R	.....	....A..T...E...R..T.INA	.....	....F.....P.	.K.DE.E-.M.....	V..T....T
H. capsulatum	..R.....K	.....A	TL.HA..L...E...R.AR.ANG	...N...S.	..Q.....D..P.	.KVDE.E-.M.....	V..T....I
B. dermatitidis	..C..I...K	.....	...HA.....D...LA.TTSDA	...N...S.	..K.....S..PQ	.KLEE.E-.M.....	...T....T

FIG 5 Amino acid substitutions in Cyp51C proteins. Numbers and asterisks show the reference positions of residues discussed in the text (blue). Vertical dashes, sequence identity between *A. flavus* NRRL3357 Cyp51C (accession number XP_002383931) and *A. fumigatus* Cyp51A (accession number AF338659); dots, residues identical to those in *A. flavus* NRRL3357 Cyp51C. Accession numbers for the other reference sequences are as follows: *A. flavus* ATCC MYA-384/AF70, KOC15064; *A. oryzae* RIB40, AB514682; *A. nomius*, XP_015411243; *A. lentulus*, AEB77687; *Trichophyton interdigitale*, EZF31978; *Trichophyton tonsurans*, EGD95049; *Trichophyton rubrum*, XP_003235929; *Trichophyton soudanense*, EZF72647; *Microsporum canis*, XP_002845046; *Histoplasma capsulatum*, EER42982; and *Blastomyces dermatitidis*, EGE84227.

H430, D435, and T496 in Cyp51B (160), and M54, S196, S240, D254, Y319, A324, N423, and V465 in Cyp51C (149, 161, 162).

As in *A. fumigatus* Cyp51A, the relevance of the Cyp51 substitutions seen in *A. flavus* must also be interpreted with respect to the variability of the respective positions in the population as well as in other fungal species.

Both the M54T and S240A substitutions in Cyp51C are present in susceptible isolates and were identified as variants in the respective studies (149, 161) only because the genome-sequenced strain MYA-384/AF70 (accession number PRJNA217045) contains a *cyp51C* isoform divergent from that of the general *A. flavus* population, including the reference genome (*A. flavus* NRRL3357 [accession number PRJNA13284]) (163). The latter also applies to the S196P, D254N, and N423D substitutions (Fig. 5). Only in the case of the M54T substitution is a known resistance substitution of Cyp51A (G54) located in the close vicinity; however, Cyp51A G54 actually corresponds to the adjacent Cyp51C position G53, not M54 (Fig. 5). At all positions except S196, variation is seen between other species. The S240A substitution (note that due to the intron in *A. flavus cyp51C*, the respective mutation is referred to as a T788G substitution; nucleotide positions of the mutations are listed in the work of Paul et al. [149]) remains particularly controversial. In one study, it was associated with a high VRZ MIC, and this phenotype was confirmed using molecular complementation assays (162). However, others have also observed it in susceptible isolates (149). Together, these observations make involvement of these residues in drug resistance unlikely (149).

Of the four remaining residues (I285, Y319, A232, and V465), only I285 shows variation within the *Aspergillus* genus (161), but the reported I285V substitution cooccurred only in isolates with other potential resistance-conferring substitutions (149).

For *cyp51A* and *cyp51B*, microevolution experiments using long-term incubation of *A. flavus* on azole-containing media have yielded strains with reduced azole susceptibility and mutations leading to multiple amino acid exchanges in both Cyp51A and Cyp51B (160). This was most prominent for Cyp51A-K197N (position K133), which was observed three times independently. Other substitutions (Table 3) were observed only once, and several less susceptible isolates with unaltered *cyp51A* and *cyp51B* sequences were also observed. Potential changes in *cyp51C* or other genes were not investigated, limiting these observations; similarly, the K133 position is not known outside these experiments to hold resistance substitutions and has not been verified independently.

TABLE 4 PCR-based assays to characterize molecular resistance azole mechanisms in *Aspergillus* spp.

PCR use and format	Target	Reference(s)
Analyses of *A. fumigatus cyp51A*		
Real-time PCR	Single regions (~100 bp)	192, 193
Mixed-format real-time PCR	TR- and amino acid substitution-specific regions	92, 194
PCR-RFLP	Multiple single regions (180–270 bp)	167
Nested PCR, two amplicons	F1, TR region to L98; F2, G54 to N266	193
Conventional one-step and nested PCR[a]	Single regions (100–173 bp) for TR_{34}/L98H and M220	175
	Single regions (103–143 bp) for TR_{46} and the L98H substitution	174
PCR with TaqMan probes	TR, G54, L98, G138, M220	83
	L98H and Y121F substitutions	71
Primer extension	TR_{34} and 15 polymorphic sites	151
LightCycler endpoint genotyping	L98H, Y121F substitutions	71
AsperGenius (PathoNostics BV, Maastricht, The Netherlands) multiplex real-time PCR and melting curve analysis[a]	TR_{34}, L98H, Y121F, T289A substitutions	25, 107, 176, 177
Real-time RT-PCR	*cyp51A* gene expression	75
Analyses of *A. fumigatus* genes other than *cyp51A*		
PCR plus sequencing	Full *cyp51B* gene in four amplicons	75
Real-time RT-PCR	*cyp51B* gene expression	75
	mdr1 and *mdr4* gene expression	75
PCR with TaqMan probes	*cyp51B* S35, P294	151
PCR plus sequencing	Full *hapE* gene	19
PCR plus sequencing, real-time RT-PCR	*cyp51A*, *mdr1-4*, *atrF*, *cdr1B* (*abcG1*), *mfs56*	17
Analyses of *A. flavus cyp51* genes		
PCR plus sequencing	*cyp51A* and *-B* genes plus 5′ and 3′ regulatory sequences	160
PCR plus sequencing, TaqMan probes	*cyp51A*, *-B*, and *-C* genes plus 5′ and 3′ regulatory sequences	162
PCR plus sequencing	*cyp51A*, *-B*, and *-C* genes plus 5′ and 3′ regulatory sequences	149

[a]Non-culture-based, direct investigation of clinical (respiratory) samples was possible after confirmation of *Aspergillus* DNA in the sample by use of diagnostic *Aspergillus* PCR assays.

How any of these substitutions affect antifungal therapy in a clinical setting remains unclear. In a nonimmunocompromised mouse model, VRZ treatment was not relevantly impaired by mutations in *cyp51C* (164). The authors of that study discussed the position of the Y319H mutation as being rather far from the binding position of azoles and a potential loss of fitness in resistant strains as explanations for the unexpected results.

Other *Aspergillus* Species

Data on azole resistance and the underlying mutations in species apart from *A. fumigatus* and *A. flavus* are scarce; however, there are 3 single observations of Cyp51A alterations reported in the literature (Table 3): the M217I substitution (position M220) in *A. terreus* (165), the R228Q substitution (position K230) in *A. niger* (166), and the L21F substitution (position L21) in *A. tubingensis* (166). The isolate with the R228Q substitution showed a PSZ MIC above the epidemiological cutoff value. For the *A. tubingensis* isolate with the L21F substitution, especially the measured ITZ MIC value of 64 μg/ml was highly elevated compared to those for other isolates (166). Only the M217I substitution corresponds to a well-known residue (M220) involved in azole resistance in *A. fumigatus*. Neither K230 nor L21 has been implicated in Cyp51A-based drug resistance, and neither observed exchange has yet been validated independently.

MOLECULAR METHODS FOR DETECTION OF ALTERATIONS AT *cyp51* LOCI

Aspergillus fumigatus

A standard method to detect alterations of the *cyp51A* gene in fungal isolates is not yet defined. The most commonly used method is simple PCR amplification of the entire coding and promoter region, followed by Sanger sequencing of the PCR products. Identification of the promoter repeat type can easily be achieved by analyzing the 5′ upstream sequence of the static repeat unit (Fig. 4B). Specific methods for detection of the TR_{34} and TR_{46} variants are also available, including several rapid TaqMan-based approaches and melting curve analyses on LightCycler instruments (Table 4). Utilization of an AluI-based restriction fragment length polymorphism (RFLP) method (167) at the

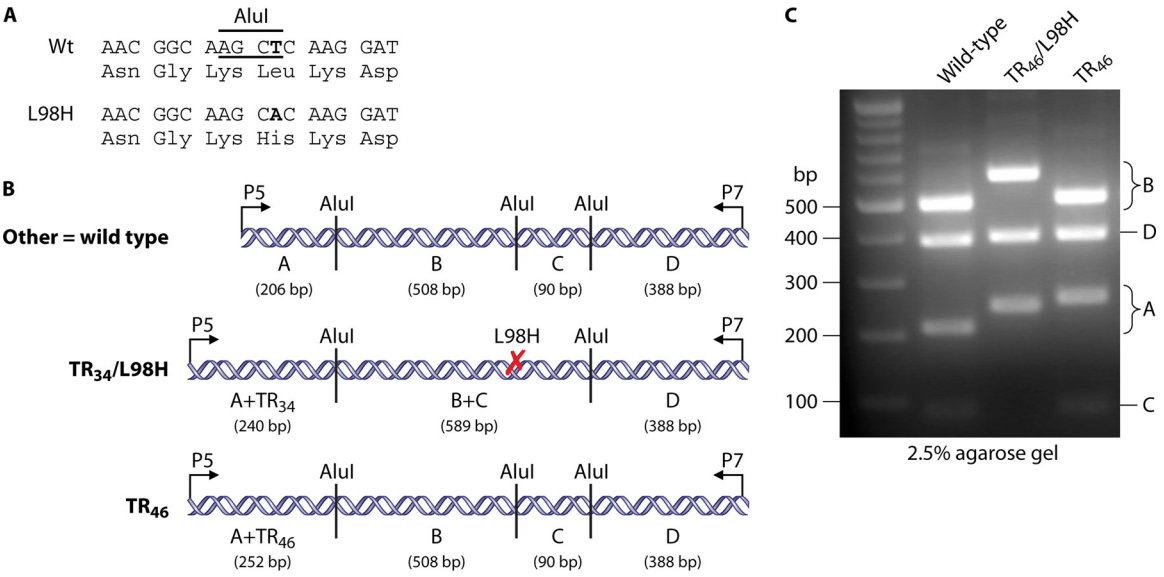

FIG 6 Interpretation of AluI-digested *A. fumigatus cyp51A* PCR product surrounding the L98 position. (A) In isolates carrying the L98H substitution, the AluI restriction site at this codon is abolished (167). (B) A PCR product amplified with the P5-P7 primer pair (16) encompasses both the L98 site and the promoter repeat region. When digested with AluI, the wild-type product is cleaved into four fragments, and the length of fragment A is characteristic of the length of the TR region. In L98H isolates, fragments B and C are not cleaved apart, resulting in a visible size shift. (C) When resolved in a high-percentage (e.g., 2.5%) agarose gel, the size shift is visualized as specific RFLP patterns for TR_{34}/L98H- and TR_{46}-carrying isolates. Other substitutions cannot be found using this method, as their RFLP patterns correspond to the wild-type patterns. The method may, however, be suitable to detect other promoter variants.

L98 codon probably has the highest value for most laboratories, as the assay is readily performed without the need for specialized equipment. It allows easy simultaneous detection of both TR_{34} and TR_{46} repeats (and likely also others), as well as the L98H substitution (Fig. 6), therefore encompassing the most frequently encountered resistance-associated *cyp51A* alleles. The commercially available AsperGenius assay also covers these two alleles (Table 4). For a few selected other exchanges at polymorphic sites, namely, G54 and M220, specialized PCR-based detection methods have been developed (Table 4).

For all other mutations, including so far unknown ones, PCR amplification of the gene in either a single (3, 18, 45, 50, 68, 82, 104, 168) or multiple (16, 44, 54, 56, 60, 62, 63, 75, 84, 100, 157, 169–171) amplicons plus subsequent sequencing is necessary. Identification of mutations in trace files or derived fasta sequences and of the respective amino acid substitutions is most easily achieved by mapping against a reference sequence. To facilitate this, we created an annotated reference containing currently known polymorphic sites along with other relevant features (Fig. 4D) (https://github.com/oliverbader/Aspergillus_fumigatus_cyp51A). Alternatively, there is also an online tool available to perform sequence analysis on *A. fumigatus cyp51A* (172).

Given the low pathogen yields by cultural methods for patients with hematological malignancies who are at high risk for invasive aspergillosis, it is very likely that azole resistance is still underdiagnosed in this population (173). The reported high mortality rates associated with ARAf underline the necessity of using non-culture-based assays for the detection of both *Aspergillus* spp. and azole resistance directly from clinical samples (24, 25, 174, 175). This approach requires higher sensitivity and lower detection thresholds than those for working on cultured isolates. Molecular methods for detecting *cyp51A* alterations directly from clinical samples have to combine high sensitivity and specificity to ensure the amplification of small amounts of *Aspergillus* DNA and to avoid cross-reactivity with human DNA. Most formats are PCR-based methods with subsequent DNA sequence analysis for the detection of mutations. PCR assays investigating primary clinical samples directly do not have the ability to amplify the whole *cyp51A* gene due to the very small amount of intact fungal high-molecular-weight DNA

in these samples. Therefore, individual PCR assays were established to amplify the key mutation-carrying gene sections. In this scenario, nested PCR assays using two consecutive PCRs amplifying the same gene region have shown the highest sensitivities. A new real-time PCR-based system for detection of *Aspergillus* DNA and the TR_{34}/L98H and Y121F/T289A *cyp51A* alterations without subsequent DNA sequencing is the AsperGenius system from PathoNostics (25, 107, 176, 177). This test system is based on *Aspergillus* DNA amplification by multiplex real-time PCR followed by melting curve analysis to detect the mutations in different PCR cycler-based detection channels. Protocols that have been used successfully directly on clinical samples (bronchoalveolar lavage [BAL] fluid and blood samples) with low pathogen DNA amounts, e.g., nested PCR (174) or multiplex PCR (25, 58, 176, 177), are summarized in Table 4. In this rapidly advancing field, prospective studies are still ongoing.

Other *Aspergillus* Species

For *Aspergillus* spp. other than *A. fumigatus*, there are currently no commonly used protocols and—to date—no commercially available kits. The best-characterized species is *Aspergillus flavus*. Molecular analysis of the *cyp51A* and *cyp51B* genes for resistance mutations was performed using PCR with subsequent DNA sequence analysis (160). In another study, amplification of *cyp51A*, *-B*, and *-C* included an additional 1 kb of genomic DNA up- and downstream of the coding regions (162). Assays for the analysis of *cyp51* expression levels by quantitative TaqMan-based real-time PCR have also been described (162). The aspects of molecular azole resistance detection in *A. flavus* are also summarized in Table 4.

OTHER RESISTANCE MECHANISMS

For a significant proportion of azole-resistant clinical *A. fumigatus* isolates, no mutations within the *cyp51A* locus are observed (16–18, 95). Compilation of the MIC values from the literature (Tables 1 and 2) showed that isolates with the VRZ resistance-conferring TR_{46}/Y121F/T289A allele fall into two clearly distinct groups with respect to ITZ cross-resistance, namely, VRZ^r $ITZ^{s/i}$ and VRZ^r ITZ^r, in the absence of other *cyp51A* mutations. We also previously observed an unusual TR_{34}/L98H isolate with an additional VRZ MIC of >32 μg/ml (39). Together, these data highlight the multifactorial ability of the fungus to overcome susceptibility, so the genetic basis for resistance is not restricted just to mutations within the *cyp51A* region. However, so far, only a few other mechanisms have been discussed to contribute to clinical azole resistance.

Most importantly, the expression of ABC transporters can be upregulated in clinical isolates (Fig. 2E); however, their contribution to azole resistance has not yet been fully clarified (17, 23). So far, the best-studied transporter in correlation with drug resistance is the ABC efflux transporter Cdr1B. This transporter shows high homology to the efflux pump Cdr1p from *Candida albicans* (*Ca*Cdr1), which is regulated by the transcription factor Tac1. Overexpression of *Ca*Cdr1 increases the resistance to fluconazole in both *A. fumigatus* (17, 21) and *C. albicans* (178). A potential regulator of Cdr1B in *A. fumigatus* that is similar to Tac1 in *C. albicans* is the zinc cluster transcription factor AtrR (179). Mutations in this regulator might provide explanations for increased resistance mediated by overexpression of Cdr1B. Nevertheless, gain-of-function mutations of either the *atrR* or *cdr1B* coding region or their promoters have not been described so far.

Data from *in vitro* approaches, such as mutagenesis, genetic backcrosses, and complementation in *S. cerevisiae*, have led to the identification of other potential drug resistance genes (19, 22, 180–182), but these have not yet been confirmed to exist in clinical or environmental isolates.

Especially when interpreting PCR and/or sequencing data in the absence of a culture isolate for susceptibility testing, it must be kept in mind that the presence of a particular mutation likely indicates resistance toward specific compounds (high positive predictive value); however, the absence of a mutation per se does not indicate susceptibility (low negative predictive value).

Standardized phenotypic tests that can be used for diagnostic procedures for detection of increased sterol biosynthesis or efflux have not yet been proposed.

CONTRIBUTION OF PHYLOGENETIC ANALYSES TO RESISTANCE DETECTION

Using genetic strain typing of a small *A. fumigatus* strain set, it has been suggested that the TR_{34}/L98H allele arose only once and has since been propagating through the *A. fumigatus* population (35). For isolates with the TR_{46}/Y121F/T289A or G54E/W allele, similar observations have been made (39, 108). This has led to only a few distinct lineages, which can be differentiated by both short-tandem-repeat typing (e.g., see references 24 and 108) and *csp1* typing (e.g., see references 39 and 170). Because of this and a significant degree of cross-resistance to agricultural fungicides, the hypothesis of an environmental origin of these alleles has been suggested (50). Strains with these alleles are thought to be propagated in the environment through the use of different azole compounds by the farming industry. Along with that proposition, an exogenous route of infection of patients has been proposed for these isolates. In contrast, isolates with other exchanges at G54 or at M220 or with one of the rarer mutations are more likely to have originated within the patient under prolonged antifungal therapy with mold-active azoles.

Short-tandem-repeat typing of *A. fumigatus* (STRAf typing) utilizes the copy number variability of nine short repeat sequences, of 2 to 4 bp in length, whose PCR amplicons are resolved by capillary electrophoresis (183). STRAf typing has a very high discriminatory power (183), and for ARAf isolates, it has been shown to readily demonstrate the genetic nearness of globally isolated TR_{34}/L98H or G54E strains (108). This makes it an excellent and robust reference method; however, capillary electrophoresis requires special equipment, which rules out its use as a rapidly established laboratory technique. Thus, the typing approach using the repetitive region of the *csp1* gene is implemented more easily (184–186). The *csp1* gene is simply amplified by PCR and sequenced, and the sequence is searched for the number and type of 12-bp-long elements. Their order gives one of (currently) 27 *csp1* types (184). As a single-locus method, it has a lower discriminatory power than that of STRAf typing, but it is more easily implemented and is practical even for a single isolate. Among the isolates tested in our own laboratory, there was a moderate correlation of TR_{34}/L98H isolates with the t04B *csp1* type, a type which has not been described for isolates with any other *cyp51A* allele (39).

Additionally, sequencing of hypervariable regions in cell wall genes ("TRESP") (187) has been shown to yield a sufficiently diverse marker set to discriminate within the *A. fumigatus* population. By this analysis, TR_{34}/L98H strains also formed a less diverse (i.e., more closely related) subpopulation.

Ultimately, whole-genome sequencing will have to reveal the phylogenetic and evolutionary relationships among early TR_{34}/L98H *A. fumigatus* isolates and their progeny. A first study (154) did indeed find similar rates of recombination between strains isolated more distantly as well as in close proximity. Similar conclusions of both local clonal expansion and global recombination were drawn from a study using STRAf typing (188).

From a clinical point of view, phylogenetic analyses are therefore interesting for identifying local outbreaks or common sources of infection, but they unfortunately have—despite a likely common ancestral origin for strains with the major resistance alleles—only low value for the prediction of azole resistance phenotypes in *A. fumigatus*.

CONCLUSIONS

The emergence of *Aspergillus* strains with azole resistance, and even pan-azole resistance, spreading through the environment rather than originating under therapy in individual patients poses a significant threat to vulnerable patient groups. Although this has not yet been shown for other alleles, most worrying is that at least Cyp51A-G54W- or Cyp51A-M220K-carrying azole-resistant *A. fumigatus* strains do not seem to suffer from growth or fitness defects (189): as in phytopathogens, azole resistance in

human-pathogenic *Aspergillus* spp. is likely here to stay. Recombination with other alleles has already been observed within *A. fumigatus cyp51A* alleles, as both TR_{34}/L98H and TR_{46}/Y121F/T289A alleles have been observed in combination with substitutions seen in the population (39, 81, 154). Since *A. fumigatus* does have a sexual cycle and genetically crosses in the wild (153, 154), it stands to reason that despite the initial clonal expansion, phylogenetic relationships between the different loci and resistance alleles will blur and eventually dissipate in the future. Within a species, the resistance traits may freely combine with each other as well as with other traits present in the wild, leading to increasingly resistant populations. Establishing the local epidemiology, for which we outlined potential workflows here, and setup of proper patient testing procedures are therefore indicated in clinical settings.

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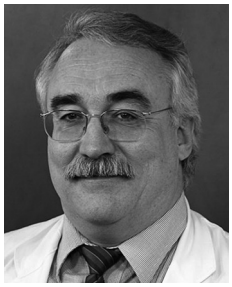

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